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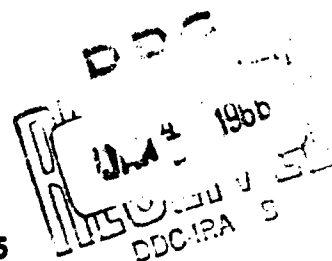
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TECHNICAL MANUSCRIPT 251

PRESERVATION OF SERRATIA MARCESCENS
BY HIGH VACUUM LYOPHILIZATION

Robert R. Dewald

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BY HIGH VACUUM LYOPHILIZATION

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Physical Sciences Division
DIRECTORATE OF BIOLOGICAL RESEARCH

Project 1B522301A08001

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ABSTRACT

Water-washed *Serratia marcescens* (ATCC strain 14041) were lyophilized in an all-glass system capable of evacuation to pressures of less than 5×10^{-6} torr. Lyophilization at the lowest pressures resulted in 50 to 65% survival for unstabilized washed organisms compared with 10 to 20% for those lyophilized at pressures of about 2.5×10^{-5} torr. At the latter pressures, 45 to 65% survivals were obtained when NaCl or Naylor-Smith stabilizer was added to the cell suspensions before lyophilization. However, the stabilizers failed to increase significantly the levels of survival compared with water suspension for those lyophilized at pressures less than 10^{-6} torr. The high survival obtained by the high-vacuum technique may be attributed to the reduction of traces of molecular oxygen that has been reported to be destructive to the dried bacteria.

Survival of unstabilized dried *S. marcescens* after 1-day storage increased markedly with decreasing sealing pressure. Under the highest vacuum attained, survival of the dried bacteria was not impaired by storage up to 1 month at dry ice temperatures, but at higher temperatures viability losses occurred. Exposure of the dried unstabilized bacteria to dry air resulted in rapid viability loss. The inactivation could be stopped almost immediately by evacuation to pressures of less than 10^{-6} torr, but the evacuation failed to reverse the viability losses that occurred during exposure.

I. INTRODUCTION

Lyophilization or vacuum freeze-drying is the most commonly employed method for preserving bacteria by desiccation.¹ This method can be considered to be a two-stage process involving first freezing and then sublimation of water from the frozen material. Death of organisms may occur during the freezing, drying, storage after drying, or reconstituting processes.

Generally, no serious problems are encountered during freezing of bacteria.² Satisfactory recoveries have been obtained with bacteria, which are even sensitive to freezing, by control of temperature, rates of cooling, and suspending media.³ Most viability losses are often considered to occur during the drying phase of the lyophilization process.⁴ A number of factors affecting survival following lyophilization also have been reported by numerous investigators.^{1,5} The suspending medium has been considered by many investigators as the most important single factor in determining the survival of an organism following lyophilization. Searches for protective substances or stabilizers that improve the survival of organisms have been highly successful and numerous substances have been reported to have protective action.^{1,6} However, Wagman and Weneck⁴ pointed out that occasionally contradictory claims are made for protective effects observed for certain stabilizers. Moreover, stabilizers that afford good protection to one organism are, at times, ineffective in protecting other organisms. The mode of action of the stabilizers is unknown, but attempts to answer this question have been recently reviewed by Heckly.¹ The addition of stabilizers limits the usefulness of the dried material for subsequent studies, but freeze-drying from distilled water suspensions results in dried organisms that are essentially pure and free from the components of any stabilizing media, allowing more valid studies. Equally important in studying the effects of various stresses on dried organisms is that the viability following drying should be high so that the dried organisms when rehydrated are characteristic of the original bacterial population.

Limited information has been reported concerning lyophilization of washed cells from water suspensions. Naylor and Smith⁵ and Zimmerman⁷ reported that poor recoveries (<5%) resulted when unfortified suspensions of S. marcescens were lyophilized. However, Lion and Bergmann⁸ reported high recoveries (50%) for Escherichia coli dried from distilled water suspensions. The latter also reported that the dried bacteria died rapidly upon exposure to oxygen and listed substances that afford protection against the lethal effects of oxygen.⁸ Electron paramagnetic resonance (EPR) studies by Lion, Kirby-Smith, and Randolph¹⁰ showed that free radicals formed during the period of rapid killing because of oxygen exposure. Similar EPR experiments by Dimmick, Heckly, and Hollis¹¹ and Heckly, Dimmick, and Windle¹² showed that free radical formation occurred for several species of dried microorganisms, including S. marcescens, when exposed to oxygen. Benedict et al.¹³ reported that lyophilized S. marcescens

were killed rapidly when exposed to atmospheric oxygen, but certain reducing agents minimized the extent of inactivation. They also pointed out that rehydration under vacuum is necessary when certain stabilizers are employed. More recently, Wagman and Weneck⁷ reported a study of the residual moisture level upon the survival of bacteria dried in the absence of stabilizers. They reported low survivals following vacuum freeze-drying, but they failed to mention whether their rehydration was performed under vacuum or after exposing the dried bacteria to air.

A number of investigators^{8,9,10,11,12} compared survival during storage in vacuo with storage in atmospheres of various gases. They all found that the survival was highest for dried organisms stored under vacuum and lowest for those stored in air or oxygen. Christian and Stockton¹⁰ studied the influence of sealing pressure on the recovery of S. marcescens and Staphylococcus aureus and noted an increase in survival when the sealing pressure was decreased.

Considering the information reported thus far, it appears that the degree of vacuum employed in vacuum freeze-drying is an important factor affecting recovery, especially when drying washed bacteria in the absence of protective additives. The primary objective of this study was to determine the importance of the degree of vacuum employed during freeze-drying S. marcescens in the absence and presence of protective substances. Other objectives were to determine (i) the effect of cell concentration on the survival following high vacuum lyophilization, (ii) the temperature dependence during storage under pressures less than 10^{-5} torr, (iii) the effect of sealing pressure upon the viability of stored S. marcescens dried from distilled water suspensions, and (iv) the effects of exposing the dried organisms to dry air followed by evacuation.

II. MATERIALS AND METHODS

A. PREPARATION OF ORGANISMS

S. marcescens (Fort Detrick, strain 8UK, ATCC strain 14041) used in study were prepared by two different methods. In one method, S. marcescens were grown and stored as frozen pellets by a process already described.⁷ Other organisms were grown for 18 hours at 25 C in 3% trypticase soy broth (BBL) fortified with 2% glucose. The harvested organisms were washed twice by alternate suspension and centrifugation in distilled water and shaken aerobically at 25 C for 3 hours. The cells were then centrifuged and resuspended in distilled water to yield suspensions containing the desired number of viable cells per ml. The washed cell suspensions were refrigerated between experiments. Viable cell populations were determined by the standard surface plating technique using nutrient agar (Difco) and incubation at 37 C for 24 hours.

B. ASSEMBLY FOR HIGH VACUUM LYOPHILIZATION

Figure 1 illustrates the vacuum assembly used in these experiments. A 3-stage oil diffusion pump permitted evacuation of the system to pressures of less than 5×10^{-8} torr as determined by an ionization gauge. The high-vacuum stopcocks were lubricated with Apiezon N vacuum grease and the standard tapers were sealed with Apiezon W wax. The condensable vapors were collected in liquid nitrogen traps. The freeze-drying ampoules were connected to the high vacuum manifold via standard taper joints. The ampoules during lyophilization were immersed in a bath containing an ethyleneglycol-water mixture held at selected temperatures within ± 0.5 C. During the final two hours of the 16 to 18 hour lyophilization cycle, the cold temperature bath was replaced with a water bath that was thermostatically controlled at 25 C (± 0.1).

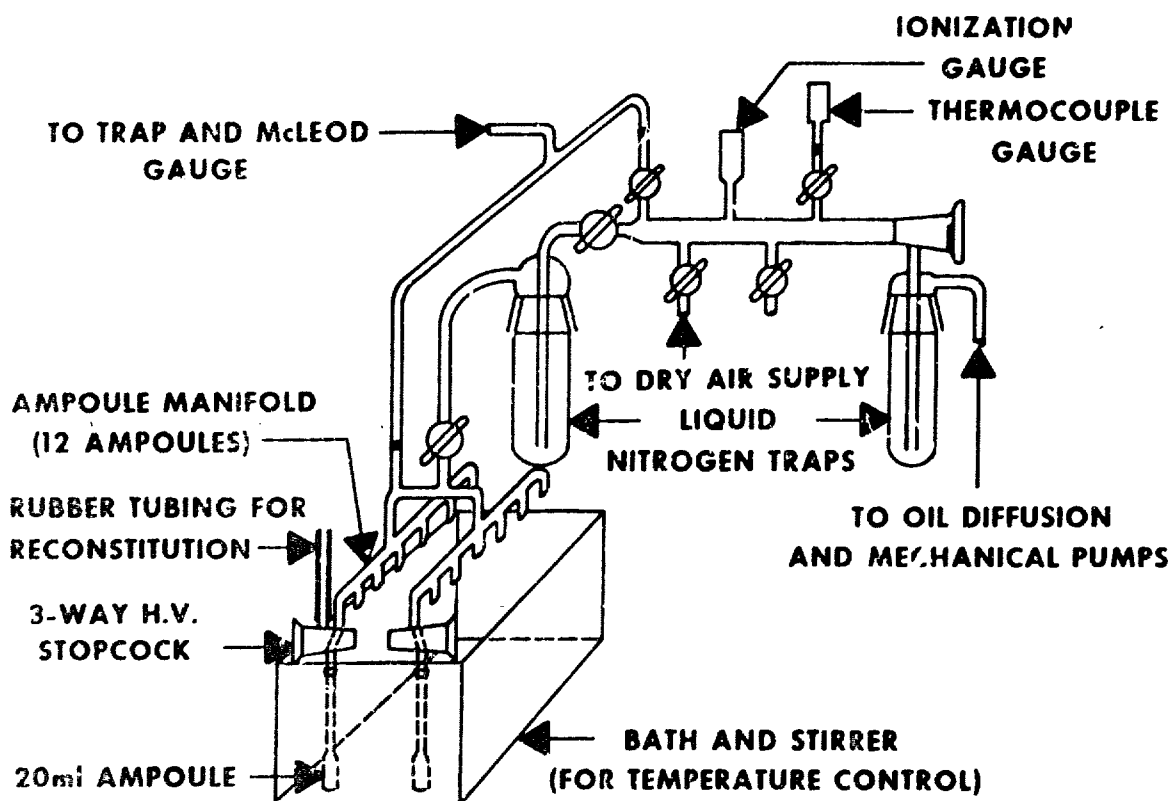


Figure 1. Assembly for High Vacuum Lyophilization.

C. LYC LIPAZATION PROCEDURE

Cell suspensions (1 ml) were introduced into 20-ml ampoules containing 4-mm-diameter glass beads. The ampoules were then connected to the high vacuum manifold and degassed by repeatedly pumping the liquid suspensions until the noncondensable gas pressure between the two liquid nitrogen traps was less than 10^{-5} torr. Next, the suspensions were frozen as a solid plug containing the glass beads by immersing them into the cold bath at -30 to -40 C. The suspensions were held at that temperature until the pressure between the liquid nitrogen traps was less than 10^{-4} torr. The frozen suspensions were then warmed to -12 to -8 C and held at those temperatures with continual evacuation at noncondensable gas pressures of less than 10^{-5} torr (approx. 14 to 16 hr). The cold temperature bath was then replaced by a 25 C bath and evacuation continued for another 2 hours. Next, ampoules were either sealed off under vacuum or kept on the high vacuum manifold during the experiments. Rehydration was accomplished by a method designed to prevent exposure of the dried organisms to the atmosphere. A piece of rubber tubing, fitted to the 3-way stopcock as shown in Figure 1 or to the neck of the sealed-off ampoule, was completely filled with water and clamped off from the atmosphere. Rehydration was then achieved by either opening the stopcock or breaking the flame-sealed tip of glass ampoules with a hammer. The amount of water added was determined by difference in weight of the ampoule containing the rehydrated cell suspension and the empty dried ampoule. The weight of the dried cells (approximately 4 to 6 milligrams) was considered negligible compared with the 2 ml of distilled water used for rehydration.

D. INFLUENCE OF DRY AIR ON SURVIVAL AFTER STORAGE

In these experiments dried air at various pressures was introduced into the ampoules before sealing. The pressures were determined with a McLeod gauge immediately before sealing. The air was dried by passing it through a trap containing activated silica gel at dry ice temperatures.

E. STORAGE EXPERIMENTS

Samples were sealed at pressures less than 10^{-5} torr and stored at room temperature, at 4 to 5 C in a refrigerator, and at dry ice temperatures. Samples were reconstituted after storage at various time intervals up to one month. The samples held under vacuum at various temperatures (-198 C to 100 C) for 1 hour under high vacuum were sealed under vacuum or left on the vacuum line during the experiment. These samples were warmed or cooled to 25 C before rehydration.

F. RESIDUAL MOISTURE MEASUREMENTS

High vacuum lyophilized samples (approx. 0.1 g) were held overnight under vacuum (2 torr) at 80 C. The moisture contents were estimated by weight difference.

III. RESULTS

To determine the optimal bath temperature for maximum survival of *S. marcescens* subjected to high vacuum drying, cell suspensions at two different initial cell concentrations were dried at various temperatures for the periods and pressures already described in the lyophilization procedure. The results of this investigation using triply washed cells are plotted in Figure 2. The temperatures correspond to those of the bath in which the ampoules were immersed during the vacuum drying process. At temperatures higher than -8 C the recovery appears to depend on the initial cell concentration. Below -8 C, where viable recovery tended to level off at its maximum value, the survival became less dependent upon initial cell concentration.

Table 1 gives a summary of viable cell recoveries obtained following vacuum lyophilization at bath temperatures between -8 and -12 C from cell suspensions of different initial concentrations. It should again be emphasized that these results were obtained from triply washed organisms in the absence of added stabilizers. In the temperature range -8 to -12 C, recoveries were essentially independent of the initial cell concentration of the suspensions. During this study, some samples of dried organisms were rehydrated with degassed water. Within the range of experimental error, no difference in recovery could be detected using degassed water for reconstitution. Also, organism suspensions prepared by the two methods yielded identical results as mentioned above.

The results obtained by lyophilization at higher pressures (using the same system without employing the diffusion pump) and at a lower pressure are given in Table 2. Also in Table 2, per cent survival of organisms dried from fortified and water suspensions are compared for two different pressures during lyophilization. The Naylor-Smith stabilizer (NS) and NaCl used in these experiments were adjusted to yield the cell-stabilizer ratios recommended by Benedict, et al.¹⁷ The results show that the protective additives had a very beneficial effect upon the level of survival after the higher pressure lyophilization, but had little effect compared with water suspensions following the lower pressure lyophilization.

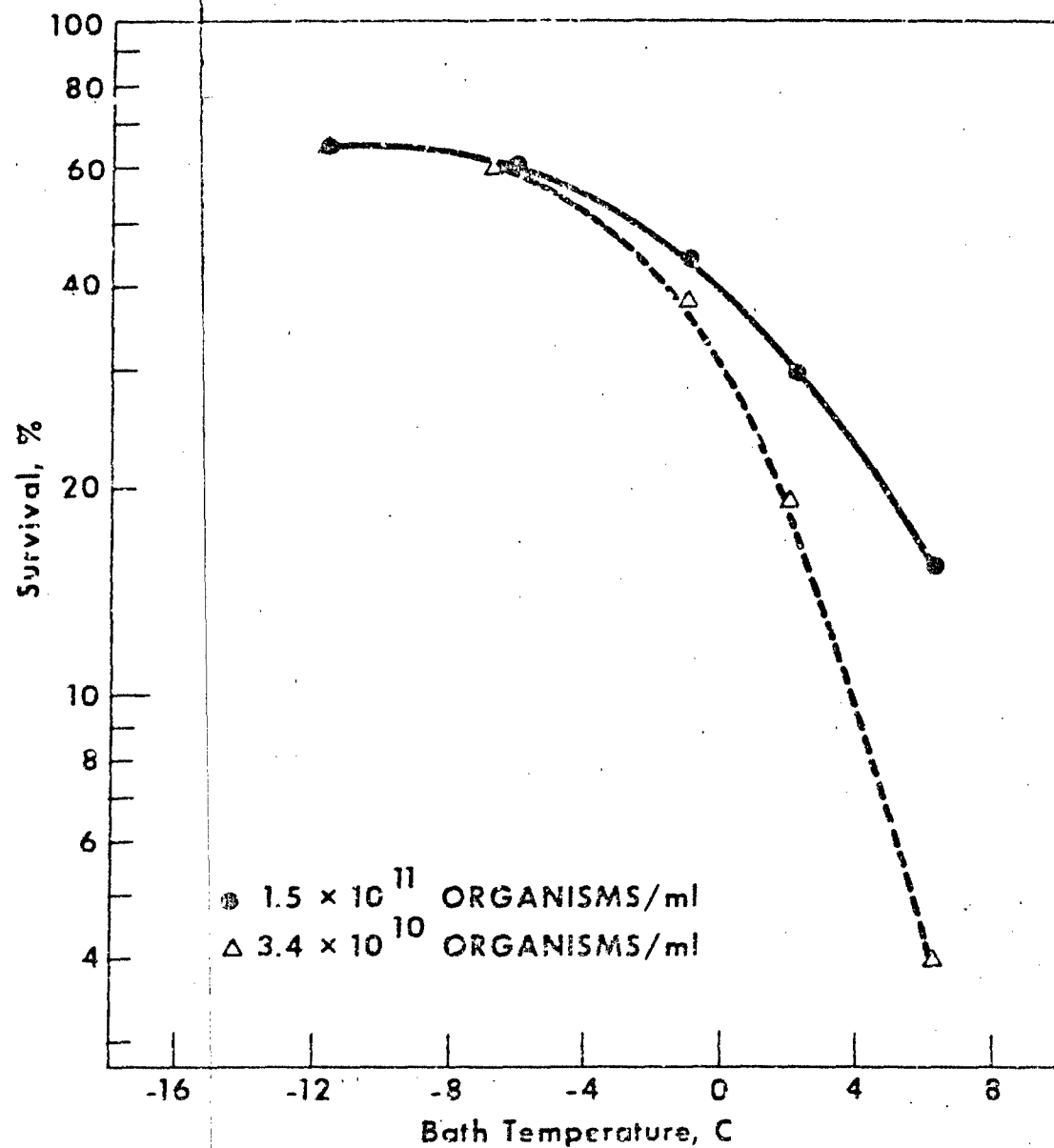


Figure 2. Semi-Log of Per Cent Survival of Two Populations of Washed *S. marcescens* Following High Vacuum Drying vs. Bath Temperature.

TABLE 1. SURVIVAL OF WASHED S. MARCESCENS AFTER LYOPHILIZATION
AT PRESSURES OF LESS THAN 10^{-2} TORR AND AFTER
FREEZING AND THAWING OF LIQUID SUSPENSIONS

Cell Concentration Before Drying, 10^9 viable cells/ml	Per Cent Survival After Lyophilization	Per Cent Freeze- Thawing Survival ^{a/}
$591 \pm 27^{b/}$	$51 \pm 7^{b/}$	$86 \pm 5^{b/}$
127 ± 12	59 ± 6	84 ± 9
16.8 ± 1.7	62 ± 9	80 ± 12
5.8 ± 0.3	53 ± 5	83 ± 5

a. Survivals for washed suspensions of S. marcescens frozen to approximately -40 C, warmed to -12 to -8 and held at these temperatures for about 16 hours, and then thawed and warmed to 25 C.

b. 95 per cent confidence interval.

TABLE 2. EFFECT OF PRESSURE DURING LYOPHILIZATION UPON THE SURVIVAL
OF WASHED AND STABILIZED S. MARCESCENS

Suspending Medium	Cell Concentration Before Drying, 10^{10} viable cells/ml	Approximate Pressure During Drying, torr	Per Cent Survival
Naylor-Smith/10	$3.9 \pm 1.3^{a/}$	2.5×10^{-2}	$55 \pm 9^{a/}$
NaCl, 0.05%	3.6 ± 0.9	2.5×10^{-2}	46 ± 6
Distilled H ₂ O	14.6 ± 0.4	2.5×10^{-2}	12 ± 4
Distilled H ₂ O	3.8 ± 0.9	2.5×10^{-2}	19 ± 3
Naylor-Smith/10	2.3 ± 1.4	5×10^{-6}	59 ± 9
NaCl, 0.05%	2.3 ± 0.4	5×10^{-6}	58 ± 9
Distilled H ₂ O	-	5×10^{-6}	$56 \pm 8^{b/}$

a. 95 per cent confidence interval.

b. Mean of values from Table 1.

Moisture determinations by the dry weight method consistently yielded moisture contents of less than 2%. No vapor pressure could be detected by an oil manometer when dried samples (approx. 0.5 g) were held at 25 C for 1 hour in a closed system. Vapor pressures greater than 10^{-2} torr are easily detected by this method. It was concluded, after comparison with reported moisture-vapor pressure data¹⁸ that the samples had little, if any, residual moisture. Therefore, data reported in Tables 1 and 2 are considered representative of samples having residual moisture content of less than 2%.

The influence of sealing pressure on survival after one day's storage at room temperature is shown in Figure 3. The survival increased markedly with decreasing pressure. This again indicates that degree of vacuum is very important for obtaining high levels of survival following lyophilization of washed organisms.

Figure 4 shows the survivals after 1 hour of storage as a function of temperature for dried organisms held at pressures of less than 10^{-5} torr. No losses in viability were detected after 1 hour at temperatures between +3 C and 32 C. When the dried bacteria were held at 0 C or below for 1 hour, 40 to 50% losses in viability occurred. Similar losses occurred when the bacteria were held at sub-zero temperatures for only 20 minutes. The dried samples originally at 25 C, cooled below 0 C, and warmed to 25 C (by immersing the ampoule in a water bath at 25 C) before rehydration, were, therefore, subjected to a cooling and warming cycle. When the bacteria were subjected to three temperature cycles, the resulting viability losses were the same as those observed after the first cycle. The viable population of the dried bacteria was reduced about 80% when held at 40 C for 1 hour. When the bacteria were subjected to temperatures between 40 and 75 C for 1 hour, the viability losses did not increase markedly with temperature, but at 100 C the losses in viability were so large that the extent was difficult to define experimentally.

The results of storage survival studies as long as one month for dried organisms sealed at pressures of less than 10^{-5} torr are given in Figure 5. At dry ice temperatures, the only viability loss appears to correlate with that found for the cooling and warming cycle previously mentioned. At 4 C, there was a gradual decrease in viability with time; the losses became more extensive at room temperature.

Figure 6 is a representation of the typical viability losses that occur when the dried organisms at 25 C are exposed to dry air at atmospheric pressure for short periods of time. In this case, approximately 85% of the dried organisms were inactivated in 15 minutes. The dried bacteria could not be reactivated by continual evacuation at pressures of less than 10^{-6} torr for periods up to 5 hours, but evacuation stopped the inactivation process immediately.

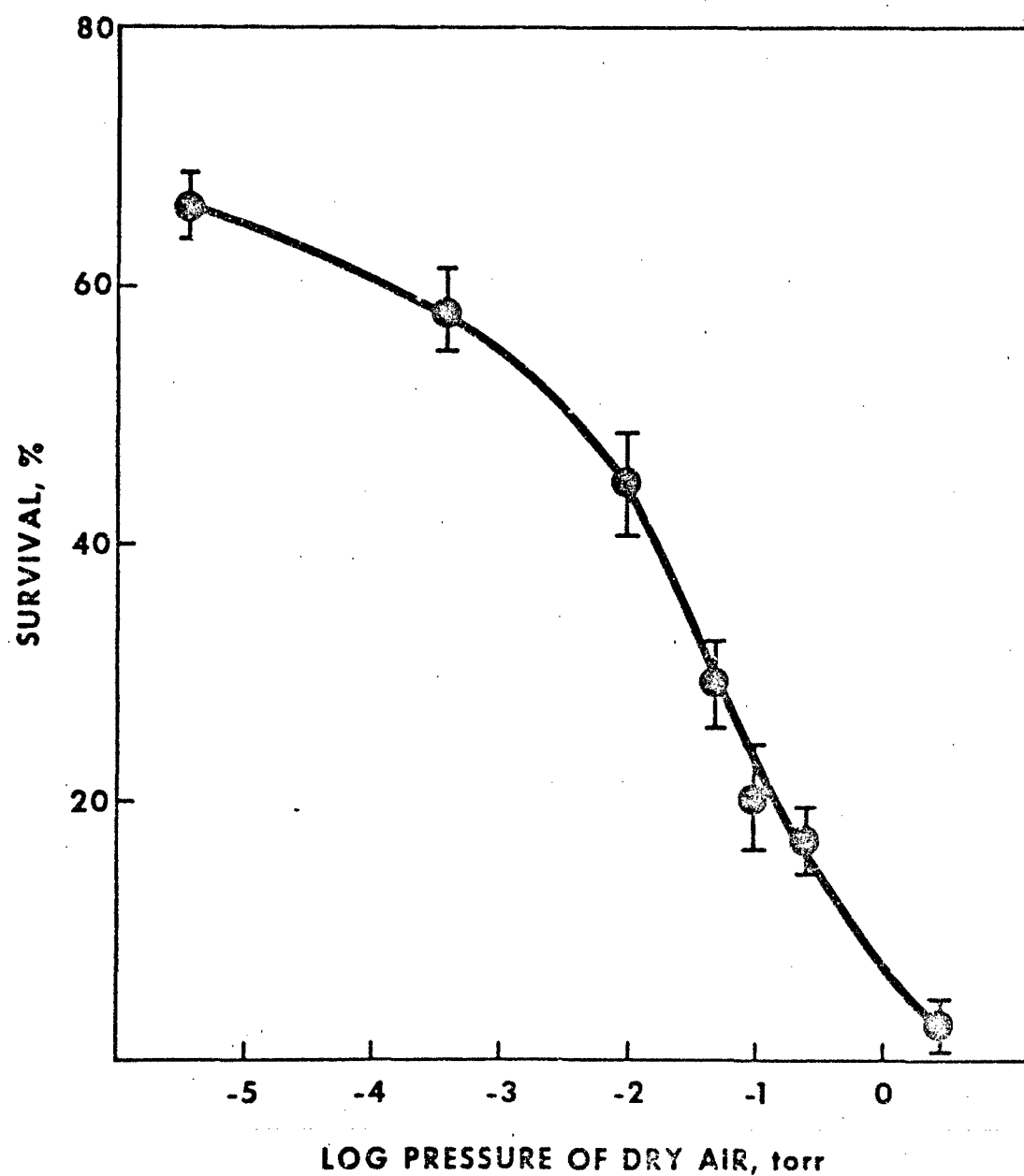


Figure 3. Survival of Dried Unstabilized *S. marcescens* after 1 Day's Storage at Room Temperature in Sealed Ampoules at Various Dry Air Pressures. Per cent survival = $N/N_0 (100)$ where N_0 and N are the number of viable organisms following lyophilization and after storage respectively.

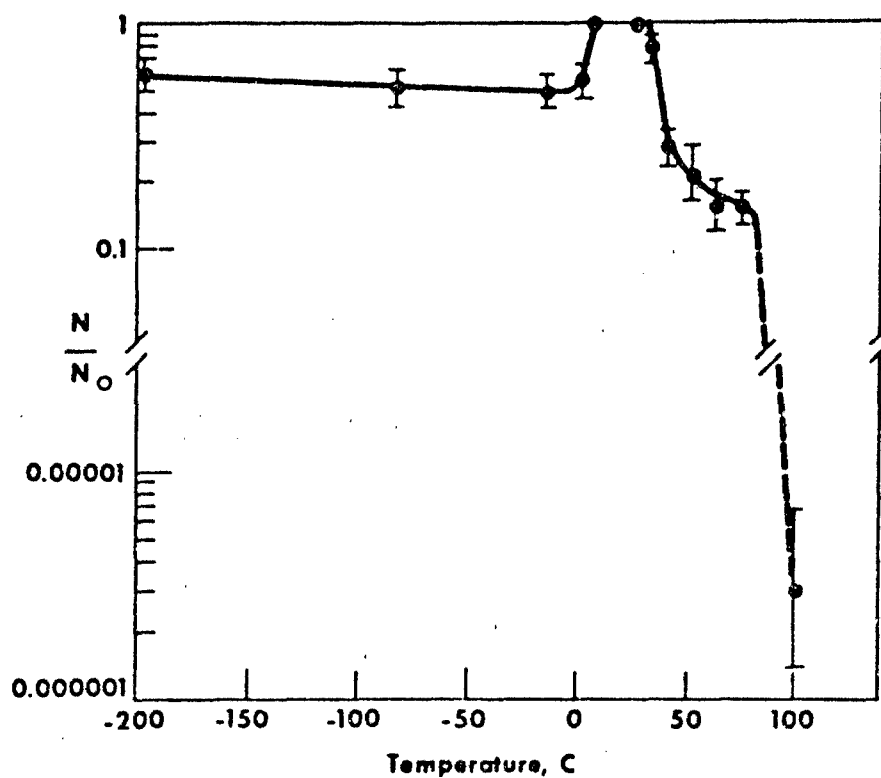


Figure 4. Semi-Log Plot of N/N_0 vs. Temperature after 1 Hour's Storage of Dried Unstabilized *S. marcescens* at Pressures of Less than 10^{-5} Torr. N_0 and N are the number of organisms before and after the storage respectively.

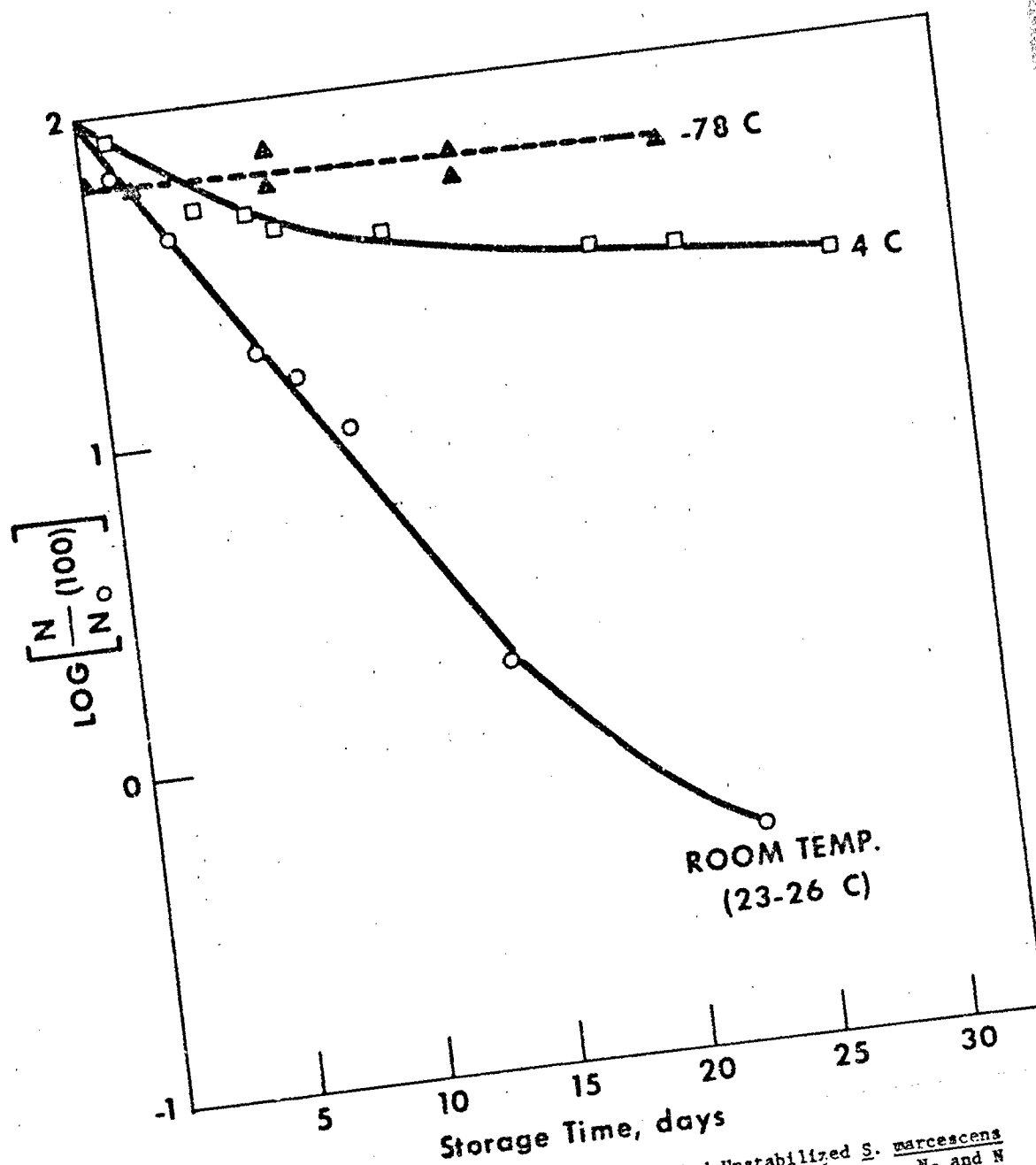


Figure 5. $\text{Log} [N/N_0 (100)]$ vs. Storage Time of Dried Unstabilized *S. marcescens* in Ampoules Sealed at Pressures of Less than 5×10^{-6} Torr. N_0 and N are the number of viable organisms immediately following lyophilization and after storage respectively.

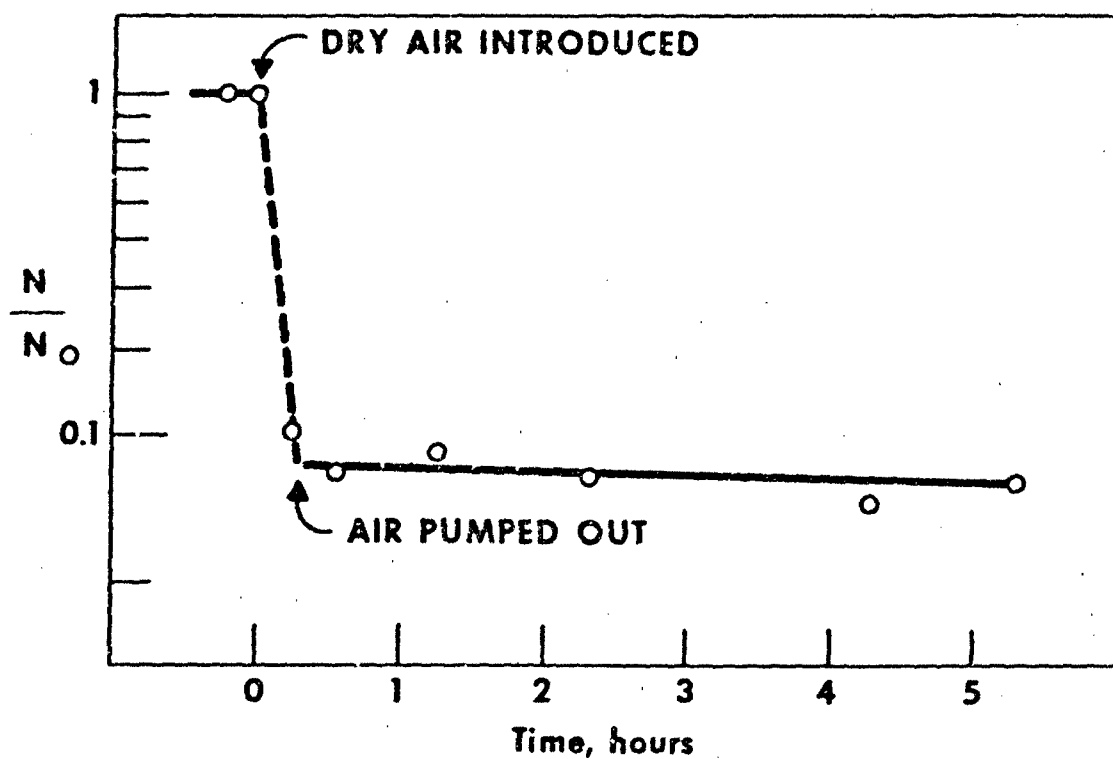


Figure 6. Semi-Log Plot of N/N_0 vs. Time for Dried Unstabilized *S. marcescens* Exposed to Dry Air for Approximately 15 Minutes and Evacuated to Less than 10^{-5} Torr. N_0 and N are the number of viable organisms before and after the exposure respectively.

IV. DISCUSSION

This study has demonstrated that high levels of survival can be obtained upon lyophilization of washed S. marcescens even in the absence of added protective substances. The pressures during lyophilization and storage were found to be important factors affecting the survival of the dried bacteria. The results obtained by the high vacuum technique can best be explained in terms of molecular oxygen, which is lethal to the dried bacteria.^{8,9,13} Also, the high vacuum appeared to have no adverse effects on the survival of the dried S. marcescens. This is in agreement with the results reported by Portner et al.¹⁸ for the exposure of dried bacteria to ultrahigh vacuums. The survivals for dried S. marcescens after 1 day of storage under various sealing pressures show that the dried bacteria are sensitive even to traces of dry air.

The results obtained from higher pressure lyophilization (approximately 2.5×10^{-2} torr) emphasize the importance of the degree of vacuum. Under identical conditions, i.e., time, temperature, and history of organisms, the higher pressure lyophilization gave substantially lower survivals: 10 to 20% compared with 50 to 65% survivals for the high vacuum technique. The addition of stabilizers (NaCl and Naylor-Smith) to cell suspensions prior to lyophilization did have a beneficial effect on the survival at the higher lyophilization pressure. However, both stabilizers failed to increase the levels of survival compared with water suspension when lyophilized at pressures less than 10^{-6} torr. Lion²⁰ suggested that perhaps the main function of stabilizers is to afford protection against the adverse effect of oxygen. The effect of pressure during lyophilization found in this study supports Lion's suggestion.

The freezing-thawing recoveries reported in Table 1 were obtained by taking samples of cell suspension through the same temperature cycle as during lyophilization except that the suspensions were not dried. The freezing, thawing, and warming of the liquid suspension consistently resulted in viability losses of 15% ($\pm 8\%$). The rate of freezing and thawing was found not to affect these viability losses. This is consistent with results reported by Heckly¹ for the same organism. The dependence of the recovery following lyophilization upon bath temperatures is also compatible with the results reported by other investigators as reviewed by Heckly.¹

The 1-hour storage studies under high vacuum gave an unexpected result below 0 C. No explanation is given for the 40 to 60% losses in viability that occurred when the dried organisms were subjected to a cooling and warming cycle. These viability losses were quite reproducible. No attempt was made to control the rates of cooling or warming in these experiments. Also, the 50 to 60% survival resulting after the cooling-warming cycle corresponds to the 50 to 65% survivals obtained for unstabilized S. marcescens after lyophilization, in which the dried organisms were subjected to the warming phase of the cycle.

Considerable viability losses occurred during storage after approximately 1 month at 25 C even under initial sealing pressures of less than 10^{-5} torr. No explanation can be given at this time concerning the nature of these losses. Naylor and Smith⁶ reported high levels of storage survival for the same organism when lyophilized in the presence of effective stabilizers. It appears reasonable that stabilizers might be beneficial for long-term storage stability even under pressures of less than 10^{-5} torr. The trend of temperature dependence upon survival during storage reported in this study is in agreement with those reported by other investigators.¹ Longer term storage survival studies are now in progress in this laboratory.

The recoveries after lyophilization reported here are in marked disagreement with those of Wagman and Weneck.⁴ They reported low recoveries for washed *S. marcescens* vacuum freeze-dried from water suspensions and higher recoveries when the organisms were freeze-dried in an atmosphere of circulating gases. They also stated that the residual moisture level was a major factor in determining the recovery of organisms and suggested that over-drying should be avoided. In the present work, high levels of recovery (greater than 50%) were obtained even at residual moisture contents of less than 2%. These results indicate that the removal of water is not the major factor causing death to organisms during lyophilization.

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<p>Water-washed <u>Serratia marcescens</u> (ATCC strain 14041) were lyophilized in an all-glass system capable of evacuation to pressures of less than 5×10^{-5} torr. Lyophilization at the lowest pressures resulted in 50 to 65% survival for unstabilized washed organisms compared with 10 to 20% for those lyophilized at pressures of about 2.5×10^{-2} torr. At the latter pressures, 45 to 65% survivals were obtained when NaCl or Naylor-Smith stabilizer was added to the cell suspensions before lyophilization. However, the stabilizers failed to increase significantly the levels of survival compared with water suspension for those lyophilized at pressures less than 10^{-5} torr. The high survival obtained by the high-vacuum technique may be attributed to the reduction of traces of molecular oxygen that has been reported to be destructive to the dried bacteria.</p> <p>Survival of unstabilized dried <u>S. marcescens</u> after 1-day storage increased markedly with decreasing sealing pressure. Under the highest vacuum attained, survival of the dried bacteria was not impaired by storage up to 1 month at dry ice temperatures, but at higher temperatures viability losses occurred. Exposure of the dried unstabilized bacteria to dry air resulted in rapid viability loss. The inactivation could be stopped almost immediately by evacuation to pressures of less than 10^{-5} torr, but the evacuation failed to reverse the viability losses that occurred during exposure.</p>		

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